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Assembly of the Hatching Envelope Around the Eggs of *Trachypenaeus similis* and *Sicyonia ingentis* in a Low Sodium Environment

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Abstract. The eggs of many penaeoidean shrimp undergo two dramatic morphological events when spawned into normal seawater (Lynn *et al.*, 1991). Following the initial release of jelly precursor from crypts in the cortex of the egg, an extracellular envelope elevates and transforms into a substantial "hatching envelope" (HE) 30–40 min after spawning. The HE's of *Sicyonia ingentis* and *Trachypenaeus similis* eggs have distinct laminar morphologies and range from 90 to 110 nm thick. The HE elevates approximately 80 μm from the egg in *T. similis* and 40 μm from the egg in *S. ingentis*. Although eggs spawned into low Na^+ artificial seawater (with choline chloride or Tris-HCl substituted for the NaCl) underwent normal release and formation of the jelly layer, the HE failed to develop normally. The HE retained a 100-nm thickness, but lacked the distinctive inner flocculent zone and dense outer covering. The HE collapsed to the egg surface, reducing the perivitelline space. Assembly of the HE resembles the formation of the sea urchin fertilization envelope and demonstrates a similar sensitivity to the lack of Na^+ in the ambient environment.

Introduction

The eggs of penaeoidean shrimp (Crustacea: Decapoda) undergo two dramatic morphological changes following their release from the female, at spawning, into the surrounding seawater (Lynn *et al.*, 1991). These changes have best been characterized in *Penaeus japonicus*, *P. setiferus*, *P. aztecus*, and *Sicyonia ingentis* and sequentially comprise: (1) the dramatic release of the jelly precursor from the extracellular cortical crypts; (2) transformation of the precursor material into a layer of jelly that forms around the egg; and (3) the elevation and assembly of the hatching envelope (HE) following the exocytosis of at least two types of vesicles from the egg cytoplasm (Clark *et al.*, 1980, 1990; Pillai and Clark, 1988, 1990). These events are initiated by contact of the egg with seawater and are independent of sperm-egg interaction (Pillai and Clark, 1987; Lynn *et al.*, 1991).

Release of the jelly precursor from eggs of at least two penaeid species, *P. aztecus* and *P. setiferus*, has been demonstrated to be dependent on the presence of Mg^{+2} in the seawater (Clark and Lynn, 1977). Once released, the heterogeneous jelly precursor is transformed into a homogeneous, translucent material surrounding the entire egg. This transformation is also reported to be dependent on extracellular Mg^{+2} (Clark and Lynn, 1977) and appears to be mediated by one or more proteolytic enzymes (Clark and Lynn, 1977; Lynn and Clark, 1987; Green *et al.*, 1990).

The next major morphological change during egg activation, assembly of the hatching envelope, has been reported in detail for *S. ingentis* by Pillai and Clark (1988). The assembly of the HE proceeds in two sequential phases at approximately 45 min post-spawning and comprises: (1) exocytosis of a group of dense vesicles that combine with a thin fluffy surface coat to produce the nascent or "thin" HE; and (2) a second exocytosis of vesicles which releases ring granules that combine with the nascent HE to form a laminar structure composed of a dense outer

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Abbreviations: HE—hat[illegible]velope; FE—fertilization envelope; ASW—artificial seawater; V[illegible]line envelope; ChCl—choline chloride; TEM—transmission electron [illegible]roscopy; SEM—scanning electron microscopy.

stratum and a more flocculent inner layer. Although the morphological characteristics of HE formation have been described elegantly for *S. ingentis*, little is known about the underlying mechanisms of its assembly around the egg. At present, no information is available concerning ion requirements or enzymatic processes involved in the transformation of the surface coat into the HE as described by Pillai and Clark (1988).

In this paper, we report the effects of Na^+ ion depletion on the morphology of HE formation in the eggs of *S. ingentis* and *Trachypenaeus similis* when sodium in the seawater is replaced with choline, Tris, or potassium. In addition, we also provide a brief comparative description of the morphological events associated with the jelly layer formation in the spawned eggs of *T. similis*.

Materials and Methods

Animals

Specimens of *Trachypenaeus similis* were collected in the Gulf of Mexico off the Louisiana coast by otter trawl and gravid females were transported to the laboratory at ambient temperature in aerated tanks under constant light. *Sicyonia ingentis* were obtained from the laboratory of W. H. Clark at the Bodega Marine Laboratory. These animals were collected off San Pedro in southern California by standard otter trawl and transported to the laboratory in chilled (4–10°C) aerated tanks. For both species, spawning was induced by placing the animals in darkness (Pillai *et al.*, 1988).

Media

Artificial normal (480 m*M* sodium) seawater (ASW) was prepared according to the formulae of Chambers and De Armendi (1979) and buffered to pH 8.3 with 10 m*M* TAPS (N-Tris[hydroxymethyl]methyl-3-amino-propanesulfonic acid). Reduced sodium seawaters (approximately 26 m*M* sodium) were prepared by substituting choline chloride in a 1:1 ratio for sodium chloride (Chambers and De Armendi, 1979), or, in some experiments, substituting either potassium chloride or Tris-HCl [tris(hydroxymethyl)aminomethane] for sodium (Gould-Somero *et al.*, 1979).

Gametes

Eggs were collected by placing a spawning female directly on top of a small beaker (30 ml beaker for *T. similis*) or crystallizing dish (30 × 75 mm) containing either control or experimental media. The cells were gently swirled to prevent sticking and clumping during the first 15–20 min after spawning. Eggs were monitored for normal elevation and appearance of HE before fixation in both controls and experimentals. In some cases, eggs originally spawned into reduced Na^+ seawater were transferred back into normal seawater at 10-min intervals through 60 min post-spawning and observed for HE formation and cleavage until 120 min post-spawning.

Hardening of the HE under different experimental conditions was evaluated by the resistance of the envelope to collapse and extraction when treated with 1.0 *M* urea (Schuel *et al.*, 1982) at 4 min and 15 min time points after the beginning of HE elevation. Eggs were observed for the beginning of the HE elevation. After the HE was first detected in 50% of the eggs observed, they were transferred at either 4 or 15 min post HE appearance into urea and were incubated for 5 min. At 5 min, the urea was aspirated off the eggs, replaced with normal seawater, and the eggs were immediately fixed for electron microscopy. Subsamples of eggs were then scored with a light microscope for the presence of elevated HEs and the remainder were processed for transmission electron microscopy for further observations.

Light and electron microscopy

Eggs were observed with light microscopy (phase and brightfield) on either a Nikon Diaphot or Nikon Optiphot microscope. A petroleum jelly ring was placed on microscope slides to prevent the coverslip from crushing the eggs placed within the ring and to reduce the effects of desiccation. Diameters of eggs and elevated HEs were measured with a calibrated filar eyepiece micrometer.

Samples for electron microscopy were fixed in either 2% glutaraldehyde in ASW, or a 1.2% paraformaldehyde/3% glutaraldehyde combination in ASW. After a 2–4 h fixation, the samples were rinsed twice in ASW followed by two rinses in 0.2 *M* cacodylate buffer, pH 7.4. Eggs were postfixed in 1% osmium in 0.2 *M* cacodylate, pH 7.4, for 1 h. Following two rinses in cacodylate buffer, the samples were dehydrated in either a graded acetone or ethanol series and embedded in Spurr's (1969) low viscosity epoxy resin or Embed 812 (EMS), respectively, for sectioning.

Samples were sectioned using a Porter Blum MT-2B or a Reichert Ultracut E ultramicrotome using either glass or diamond knives. Thick sections (1 μm) were stained with 0.5% toluidine blue and observed with a light microscope. Thin sections were stained with methanolic uranyl acetate and aqueous lead citrate and observed with a JEOL 100-CX transmission electron microscope (TEM).

For scanning electron microscopy (SEM), eggs were processed as described above for transmission electron microscopy except that after dehydration with the graded acetone series, the eggs were critical point dried, mounted on SEM stubs with double stick tape, and coated with 10 nm gold. Samples were then viewed with either a Hitachi HS-500 or a Cambridge StereoScan 260 SEM.

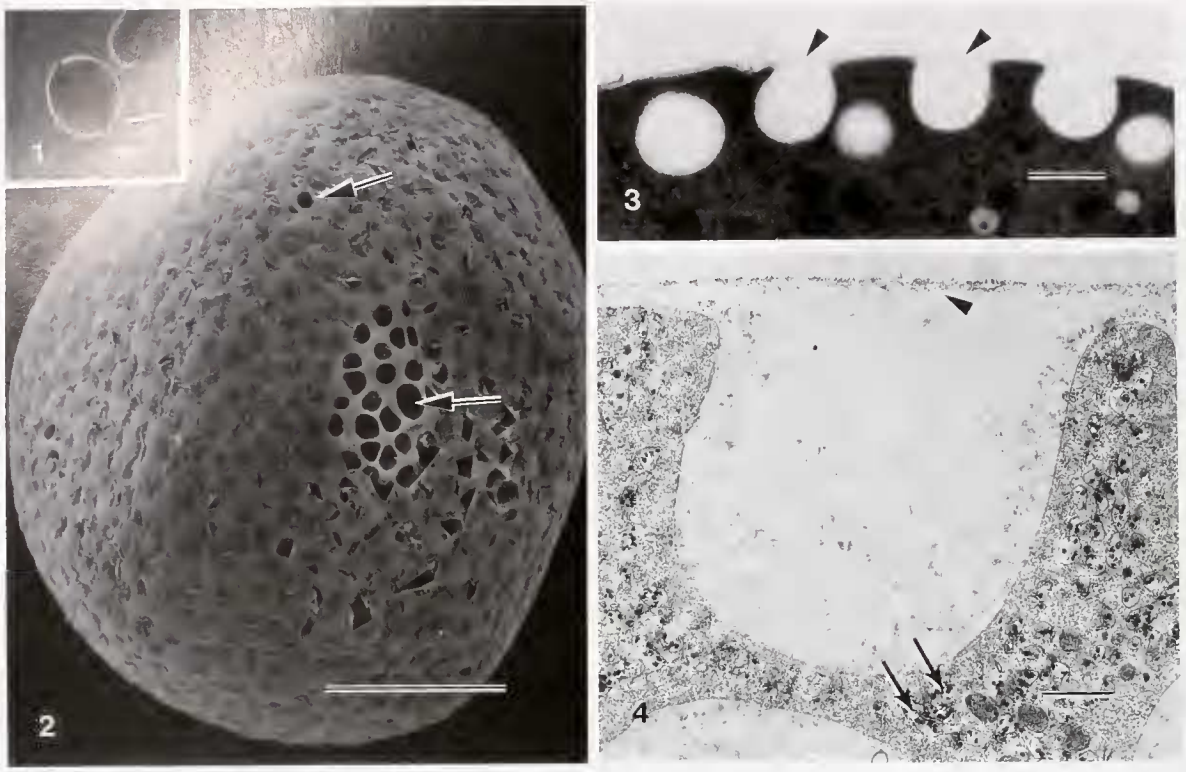


Figure 1. A phase micrograph of a *Trachypenaeus similis* egg spawned directly into seawater containing fixative. Note birefringence of the cortical region of the egg (arrowhead) and the irregular egg shape. Bar = 100 μm.

Figure 2. The vitelline envelope overlying the egg plasma membrane and cortical crypts of *T. similis* are apparent in this SEM. Note the openings of the underlying crypts where the vitelline envelope has been disturbed (arrow) and their non-uniform diameters. Bar = 50 μm.

Figure 3. The relationship between the thin vitelline envelope (arrowheads) and the underlying cortical crypts can be observed in light micrographs of thick plastic sections. Bar = 10 μm.

Figure 4. In this TEM of a *T. similis* egg crypt, the jelly precursor material appears as a diffuse fibrillar material. The egg cytoplasm contains cisternae (arrows) with electron-dense material. The vitelline envelope is a thin, fluffy material overlying the crypts (arrowhead). Bar = 1 μm.

Statistical analysis

Comparisons of dimensional changes of total egg + hatching envelope diameter between control and experimental samples were performed using Instat (Graphpad Software, Inc.) for the Student's *t*-test using a two-tailed test with test for significance of $P < 0.001$. Number of trials for each comparison are given as n.

Results

Morphology of T. similis egg activation

Although the morphology of the early activational events observed in spawned eggs of *Trachypenaeus similis* is very similar to that described in *Penaeus sp.* and *Sicyonia ingentis* (i.e., Clark *et al.*, 1980, 1990; Lynn *et al.*, 1991), a description is provided here for the first time to illustrate the similarities and the minor differences. Eggs of *T. similis* spawned into seawater and fixed within 1 min are approximately 220 μm in diameter. A bright refractile ring in the cortex of the egg corresponds to the cortical crypts that contain the jelly precursor material (Fig. 1). Release of the jelly precursor from the cortical crypts and transformation of the precursor into a homogeneous jelly layer around the egg of *T. similis* closely resembles that of *P. aztecus* and *S. ingentis*. The crypts are separated from the external environment only by a thin investment coat, the vitelline envelope (VE) (Figs. 2, 3, 4). It should be noted, however, that the jelly precursor in *T. similis* does not appear to have the small feathery elements previously described for other penaeid species (Clark *et al.*, 1980, 1990; Clark and Lynn, 1977; Lynn and Clark, 1987; Lynn *et al.*, 1991). Each crypt is partially encompassed by a granular cytoplasmic zone devoid of yolk platelets (Fig. 4). The peripheral cytoplasm also includes

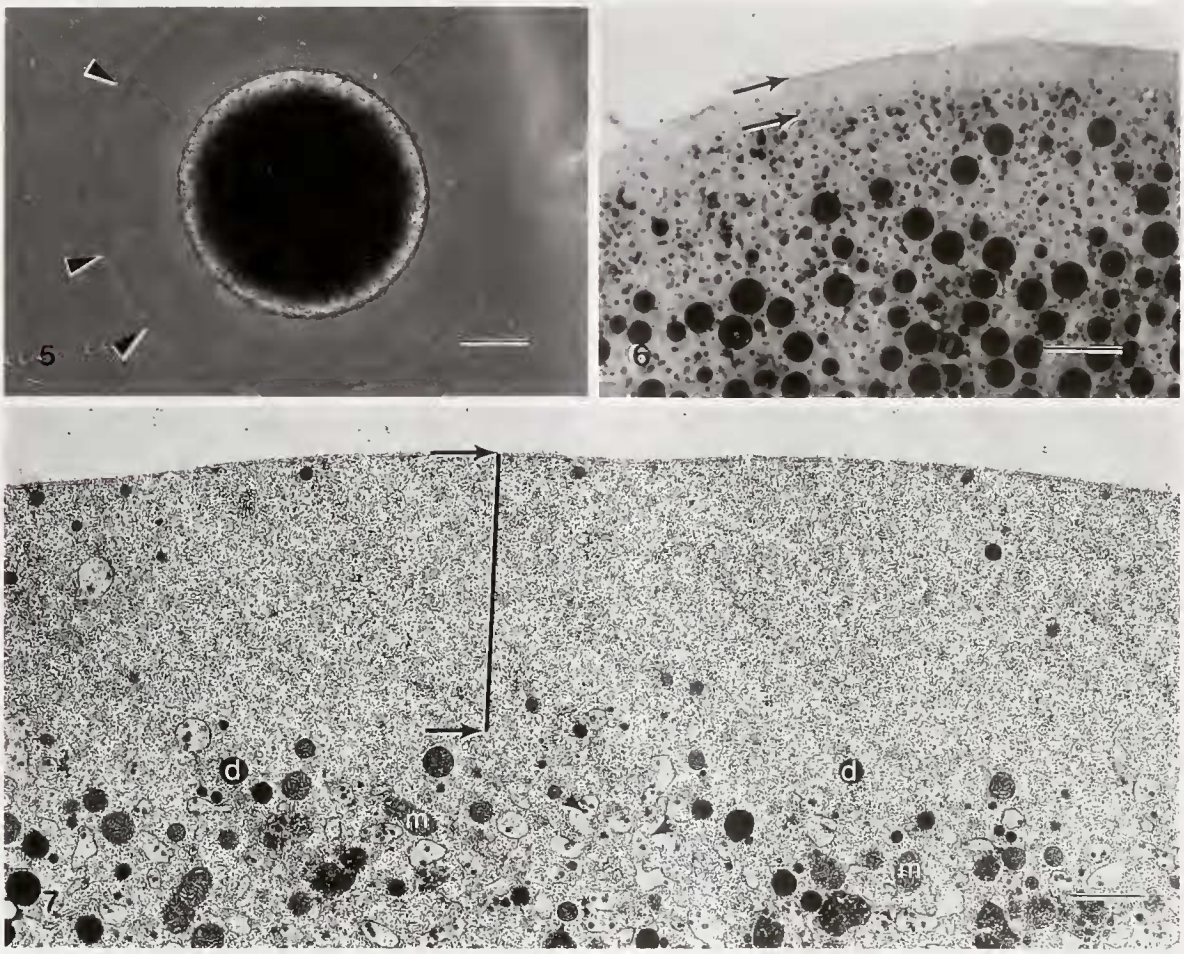


Figure 5. A phase micrograph of a living *Trachypenaeus similis* egg 6 min post-spawning reveals the translucent jelly layer (arrowheads) formed from the cortical crypt material. Bar = 50 μm.

Figure 6. At 30 min, the *T. similis* egg has a distinct cortical cytoplasmic band (delimited by arrows) observable in light micrographs of thick plastic sections. This band separates the cell membrane of the egg from the central cytoplasm which contains yolk platelets and darkly staining vesicles. Bar = 10 μm.

Figure 7. As observed at higher magnifications in TEM micrographs, the cytoplasmic band (delimited by arrows with bar) in the cortex is devoid of major large organelles. The underlying cytoplasm contains mitochondria (m), dense vesicles (d), and cisternal elements (arrowheads) with densely staining granules. Bar = 1 μm.

large numbers of cisternae which contain granules. These vesicles extend into the deeper cytoplasm as well.

Immediately upon contact of the eggs with seawater, the jelly precursor is released from the crypts and, within a few minutes, is transformed into a translucent, homogeneous layer around the egg (Fig. 5). The release of the jelly precursor from the crypts occurs within 1–2 min, but its transformation into a homogeneous layer may require up to 5 min at 21–22°C. The precursor material released from the cortical crypts is granular and heterogeneous as reported for the dispersal phases of other penaeid species, even though *T. similis* lacks the distinct feathery elements as noted above. During this time the surface of the egg rounds up and smooths out (Fig. 5). A granular band of cytoplasm remains evident in the cortex for 30–35 min post spawning and contains no major organelles (Figs. 6, 7). As also reported for *S. ingentis* (Pillai and Clark, 1987), the *T. similis* egg is spawned in first meiotic metaphase arrest. Between 25 and 30 min, the first polar body is released (not shown) and precedes the elevation of the HE.

The translucent jelly layer remains around the egg for an extended period of time and is still visible when the HE is elevated between 35 and 45 min post-spawning. The elevation of the envelope is virtually simultaneous around the entire egg and does not appear to proceed in

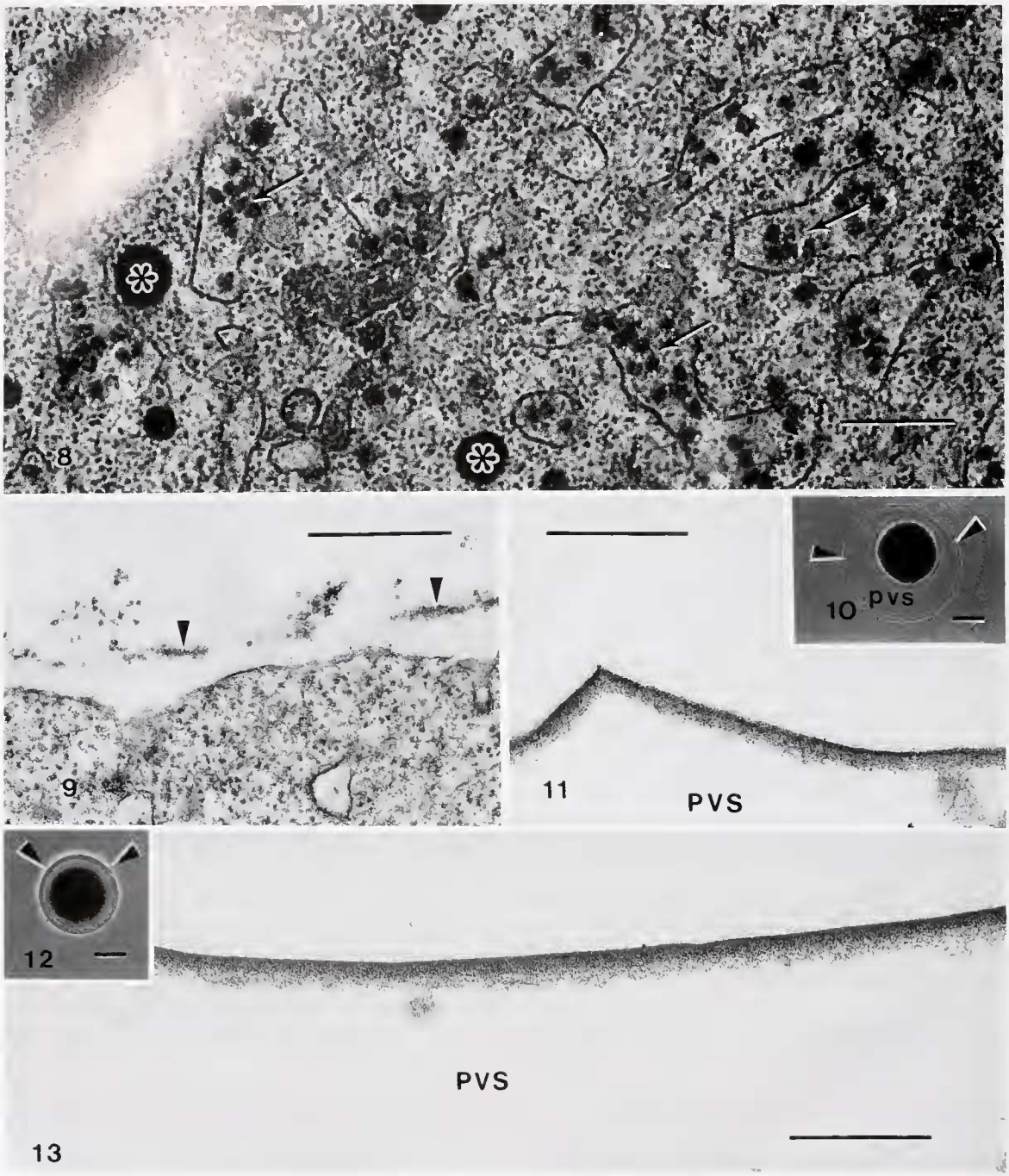


Figure 8. Both ring-like inclusions (arrows) sequestered in cisternal elements and dense granules (asterisks) are observed in higher magnification TEM micrographs of the deeper non-cortical cytoplasm of *Trachypenaeus similis* eggs. Bar = 0.5 μm.

Figure 9. In this TEM micrograph of a *T. similis* egg, an early HE has an amorphous structure (arrowheads) prior to the release of the ring vesicles from the cisternal elements. Bar = 0.5 μm.

Figure 10. By about 60–75 min, the formation of the HE is complete in *T. similis*. A phase micrograph shows the large perivitelline space (pvs) that is formed and the refractile character of the elevated HE (arrowheads). Bar = 100 μm.

Figure 11. A late or "tanned" HE of *T. similis* observed with TEM shows a distinct laminar structure consisting of a flocculent inner layer facing the perivitelline space (PVS), and a dense outer layer. Bar = 0.5 μm.

Figure 12. A phase micrograph of an *S. ingentis* egg with HE (arrowheads) at 60 min. Bar = 100 μm.

Figure 13. A TEM of the "tanned" HE of *S. ingentis* demonstrating the distinct laminar structure. As in the *T. similis* HE, a flocculent inner layer faces the perivitelline space (PVS) and lies beneath a dense outer covering. Bar = 0.5 μm.

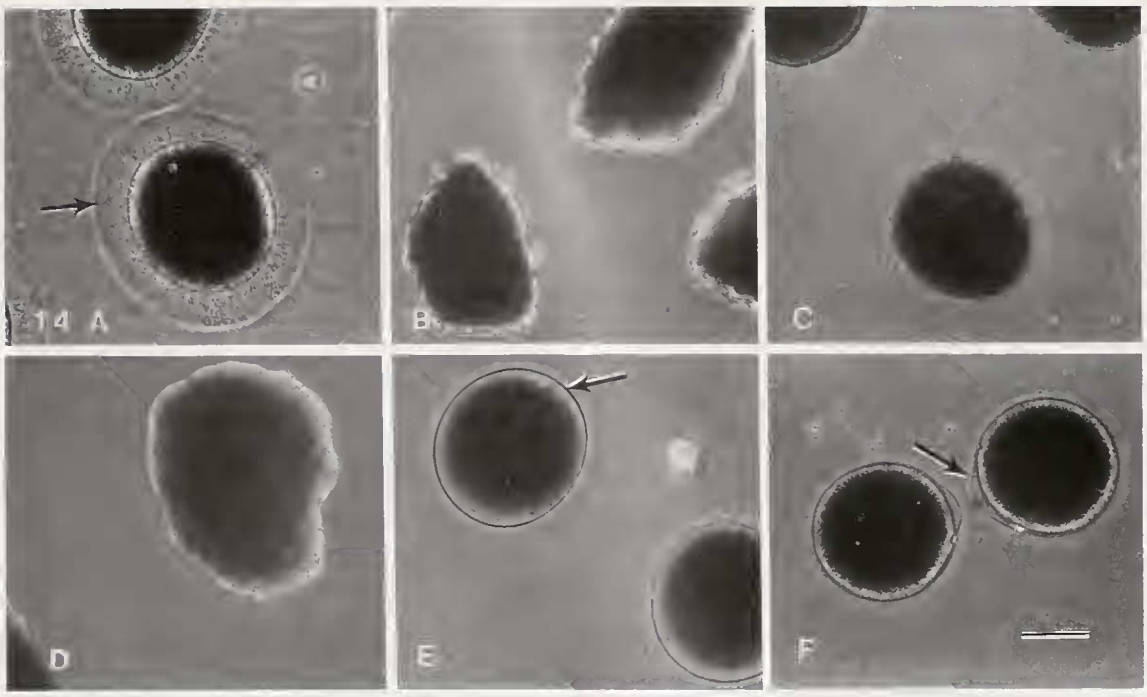


Figure 14 A–F. A series of phase micrographs of living eggs showing the altered formation of the HE in both *Trachypenaeus similis* and *Sicyonia ingentis* in reduced Na^+ seawater. (A) A *T. similis* egg with jelly coat (arrow) in ChCl-substituted seawater at 4–5 min post spawning. (B) A crenated *S. ingentis* egg in ChCl-substituted seawater at 12 min. (C) A *S. ingentis* egg has rounded up in ChCl-substituted seawater by 35 min showing a typical morphology for both species. (D) A phase micrograph at 20 min of a *S. ingentis* egg spawned into Tris-substituted seawater showing the prolonged irregular morphology of eggs in this media. (E) The collapsed HE (arrow) around the *S. ingentis* egg in ChCl-substituted seawater at 65 min. (F) Collapsed HEs (arrow) around *T. similis* eggs in ChCl-substituted seawater at 65 min. Bar = 100 μm for all phase micrographs.

a wave-like fashion. Two populations of granules also have been identified in the *T. similis* egg (Fig. 8) and appear similar to the dense and ring vesicles in the *S. ingentis* egg (Pillai and Clark, 1988; Lynn *et al.*, 1991). The dense vesicles migrate to the cortex and line up just beneath the plasmalemma by 30 min post spawning. This population of vesicles is the first to undergo exocytosis. Subsequently, the ring vesicles contained in cisternal elements migrate to the cortical cytoplasm and undergo exocytosis at a slightly later time.

The morphology of the HE formation around *S. ingentis* eggs has been previously reported by Pillai and Clark (1988). The formation of the HE around *T. similis* eggs is similar. It progresses from a fibrous, non-laminar structure (Fig. 9) to a well defined bilayered structure (Fig. 11). By 60 min post-spawning, the HE consists of a dense outer stratum covering a thicker more flocculent inner layer (Fig. 11) similar to that observed in *S. ingentis* (Fig. 13). A space between the oolemma and the elevated, hardened HE is distinct and defines the perivitelline space (Figs. 10, 12).

Effect of low sodium seawater on HE formation

Eggs of both *T. similis* and *S. ingentis* spawned into and held in seawater with choline chloride replacing sodium chloride underwent normal, early morphological changes as described above. The egg jelly precursor was released from the crypts and transformed into a translucent jelly layer as in normal seawater (Fig. 14A). In contrast to control eggs in normal seawater, the egg surface remained crenated in *S. ingentis* eggs (Fig. 14B), and the overall morphology was irregular and flattened. The eggs eventually rounded up by approximately 25 min in the choline chloride-substituted seawater (Fig. 14C). First polar body formation was delayed by approximately 10 min in both *T. similis* and *S. ingentis*.

The elevation of the HE characterizing the later morphological events was significantly reduced in eggs of both *T. similis* and *S. ingentis* spawned into ChCl-substituted seawater. Observed with light microscopy, the HE did not fully elevate in either species and remained conspicuously close to the surface of the egg (Fig. 14E, F). Measurement of the total diameter of the egg and

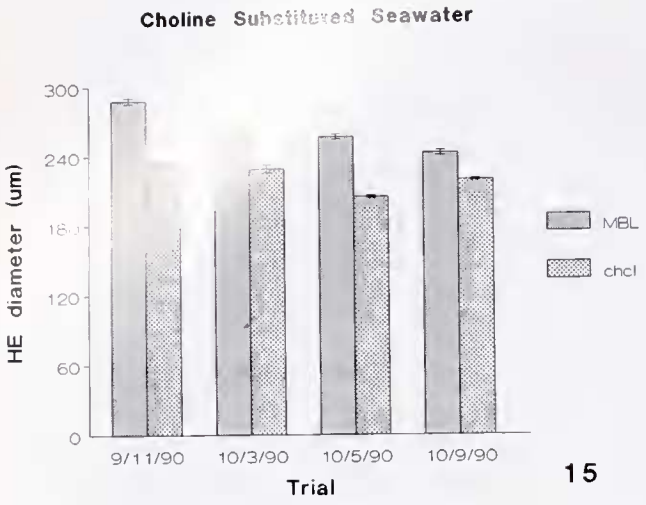


Figure 15. Comparison of hatching envelope diameters of eggs spawned into ASW or choline chloride substituted seawater. In each of the four trials, HE diameters were significantly less in choline chloride substituted seawaters (error bars are standard error; $P < 0.001$; Student t-test).

HE in either ASW or ChCl-substituted seawater revealed a statistically significant difference ($P < 0.001$, Student *t*-test between controls and experimentals (Fig. 15). In addition, the perivitelline space was not as well defined as in eggs spawned into normal seawater (compare Figs. 10, 12 and 14E, F). The thickness of the HE as observed with transmission electron microscopy remained approximately the same, but the definitive bilayered appearance was absent even as late as 70 min after spawning (Figs. 16, 17).

Tris-HCl-substituted seawater prevented the irregular egg surface from smoothing out for an extended period of time in *S. ingentis* (Figs. 14D, 18). Although jelly transformation appeared normal, polar bodies were not observed and the HE did not elevate as late as 90 min post spawning. Envelopes were not detected with TEM, and numerous ring vesicles remained in the cytoplasm as late as 60 min post-spawning.

In contrast, potassium-substituted seawater had little, if any, effect on the spawned eggs of *S. ingentis*. Jelly precursor release was normal, and transformation to a translucent layer was complete. Polar bodies were observed and the HE elevated with no delay as in the normal seawater controls. The bilayered appearance of the envelope in electron micrographs was similar to that observed in the control eggs (Fig. 19).

Transfer from choline chloride-substituted seawater back to normal seawater rescues HE formation

S. ingentis eggs spawned into ChCl-substituted seawater and transferred to normal seawater at varying times, elevated a normal HE as long as the transfer preceded the beginning of HE formation. In *S. ingentis*, transfers between 30 and 45 min post-spawning resulted in eggs with HE elevations not significantly different from controls ($P < .001$, 7 of 9 trials). Although results were assayed only at the light microscopic level, elevated envelopes appeared identical to control eggs in normal seawater. Hatching envelopes of eggs transferred back into ASW once HE elevation had begun (times greater than 50 min post-spawning) were not significantly different from eggs held chronically in ChCl-substituted seawater ($P < .001$, 3 of 3 trials).

In reciprocal experiments, eggs initially spawned into ASW and transferred into ChCl-substituted seawater at times earlier than approximately 45 min (prior to the initial observation of an elevating HE), all had HE's which were significantly reduced in their elevation compared to control counterparts (n = 3 trials). Later transfers did not appear to affect HE elevation.

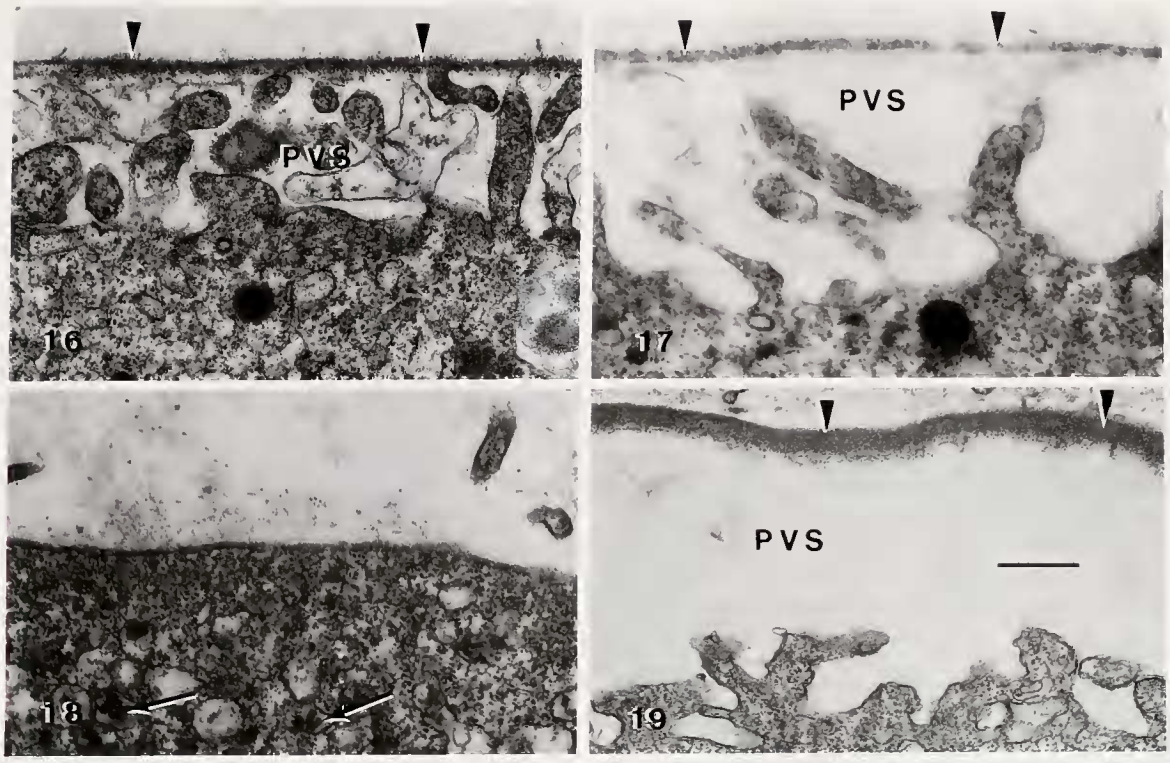
Urea disperses HEs elevated in low sodium but not those in normal seawater

Eggs of *S. ingentis* spawned into ASW were subsequently treated for 5 min with urea at either 4 or 15 min after the initial elevation of the HE was detected at the light level. At 4 min, the HE's were noticeably less birefringent, more wrinkled in appearance, and of lower elevation when observed with light microscopy. Observations with TEM revealed that at 4 min the outer dense stratum was still present, but the inner flocculent layer was not present (Fig. 20). By 15 min, the envelopes were still wrinkled, although the birefringence of the envelope appeared similar to controls when observed with light microscopy. TEM observations demonstrate that a distinct laminar structure had developed although the inner layer was slightly more flocculent in appearance (Fig. 22) than eggs spawned into normal seawater.

The HEs of eggs spawned into ChCl-substituted seawater were significantly affected when treated with urea at both 4 and 15 min after the elevation of the envelope was first detected. The envelopes in both cases were almost always indistinguishable from the surface of the eggs in phase contrast microscopy. In electron micrographs, the HEs appeared as broken investments with a fluffy irregular morphology (Fig. 21). Those envelopes treated with urea at the 15 min time point were less irregular but retained a distinct fluffy appearance (Fig. 23). In neither case was a laminar morphology ever observed.

Discussion

Eggs of several species of penaeoidean shrimp undergo similar morphological events associated with egg activation following the release of the eggs into normal seawater.



Figures 16–19. A series of TEM micrographs showing effects of various substitutions on HE formation. Arrowheads = HE; PVS = perivitelline space. Bar = 0.5 μm for all.

Figure 16. A *Trachypenaeus similis* egg at 60 min showing the fluffy appearance of an incomplete HE in ChCl-substituted seawater.

Figure 17. A *Sicyonia ingentis* egg at 70 min showing the fluffy appearance of an incomplete HE in ChCl-substituted seawater.

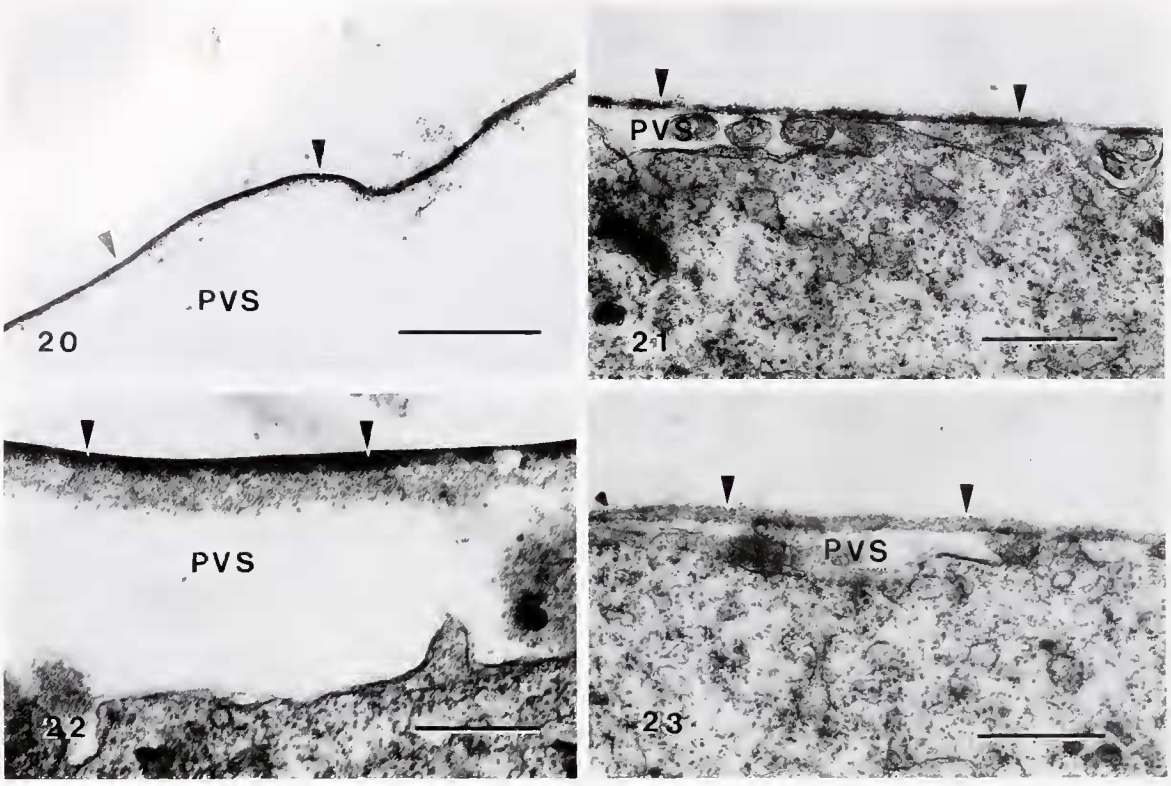
Figure 18. A *S. ingentis* egg at 60 in Tris-substituted seawater. Note the ring vesicles (arrows) in the peripheral cytoplasm. HEs were not detected in any samples.

Figure 19. A *S. ingentis* egg at 60 min in KCl-substituted seawater. The morphology for these HEs appears normal.

Particularly notable are two dramatic and distinct extracellular events resulting in the assembly of new coats around the egg (Clark *et al.*, 1980, 1990; Lynn and Clark, 1987; Pillai and Clark, 1988). The results reported here are the first to demonstrate a role for sodium in the elevation or hardening of the hatching envelope in the penaeoidean shrimp species *T. similis* and *S. ingentis*. Sodium can be replaced by potassium but not by Tris or choline, classic substitutes for sodium (*e.g.*, Chambers and De Armendi, 1979; Schuel *et al.*, 1982). The major effect on the morphology of the HE related to the deficiency of sodium appeared to be the incomplete transformation of the nascent envelope into a definitive bilayered pattern. Particularly interesting is the fact that both of these penaeoid species show a similar dependence on the presence of sodium ions for the assembly of the HE.

A sodium dependency is also observed in the elevation and hardening of the sea urchin fertilization envelope (FE) (Nishioka and Cross, 1978; Schon and Decker, 1981; Schuel *et al.*, 1982; Schuel, 1985; Cheng *et al.*, 1991). The HE of the shrimp and the FE of the sea urchin are similar in that they are formed as the result of the exocytosis of cortical granules, both envelopes are built on a template of a preexisting "surface coat," and both serve a protective function during later developmental stages (Schuel, 1978). During assembly of the FE in the sea urchin, *Strongylocentrotus purpuratus*, the microvillar casts in the vitelline envelope are transformed from a blunt shape to an angular shape apparently by the insertion of structural proteins into the fertilization envelope during the hardening process (Carroll and Baginski, 1978; Chandler and Heuser, 1980; Kay and Shapiro, 1985).

Crosslinking of the components in the hatching envelope were assayed by solubility of the envelope or its components in urea. These assays were similar to those performed by Schuel *et al.* (1982) on sea urchin fertilization envelopes. Several differences between these two systems were apparent from our current observations. First, in the



Figures 20–23. A series of TEM micrographs of *Sicyonia ingentis* eggs spawned into ASW or ChCl-substituted seawater, treated at either 4 or 15 min post HE elevation with 1 *M* urea for 5 min, washed and prepared for EM. Arrowheads = HE; PVS = perivitelline space; Bar = 0.5 μm for all.

Figure 20. ASW at 4 min. Dense outer layer remains, but inner flocculent layer is absent (compare to Fig. 13).

Figure 21. ChCl-substituted seawater at 4 min. The HE is fluffier than the dense outer layer remaining in Figure 20 and collapsed onto the egg surface.

Figure 22. ASW at 15 min. The HE retains the normal morphology of a dense outer stratum over an inner flocculent layer.

Figure 23. ChCl-substituted seawater at 15 min. The HE remains fluffy and collapsed onto the egg surface. There is no distinct morphological difference between the ChCl-substituted, urea-treated HE at 4 min and this later time point.

shrimp egg, the envelope was not completely removed with urea in either normal or ChCl-substituted seawater. Therefore, the exocytosis of the dense granules combining with the pre-existing surface coat formed a very stable HE template ("thin HE" of Pillai and Clark, 1988) within 4 min of its appearance. This initial stage of HE formation, then, appears to be independent of the absence of sodium or the presence of choline. In contrast, the fertilization envelope of sea urchins remains labile to urea solubilization for up to 10 min in both normal and sodium-depleted seawaters, indicating the time required for effective crosslinking (Schuel *et al.*, 1982).

Second, HEs of shrimp eggs in normal seawater at 15 min were not affected by urea. Both the outer dense and inner flocculent layer appeared to be intact, implying that the insertion and crosslinking of ring vesicle material into the outer dense layer was completed (Pillai and Clark, 1988). In contrast, the inner layer of HEs in sodium-depleted seawater did not form. In sodium-depleted seawater the thin HEs appeared similar whether treated with urea or not. This suggests that insertion of the ring vesicle material depends upon the presence of sodium (or potassium).

These observations (consistent with those of Schuel *et al.*, 1982, 1985) implicate sodium in the integration of structural proteins into the HE of shrimp eggs. Moreover, in the sea urchin system, quaternary amines affect the elevation of the fertilization envelope, possibly by interfering with protein insertion into the nascent envelope (see Kay and Shapiro, 1985, for review). Although quaternary amines have not been investigated systematically for their effects on the elevation and hardening of the HE around penaeoid eggs, we cannot rule out the possibility of similar effects of the amine-containing compounds, Tris

and choline. It should be pointed out that in the present studies, of the substitutes used, only choline is a quaternary amine. In addition, in experiments with a partial choline chloride substitution for sodium HE elevation was not statistically different from control eggs. These data suggest that concentrations of choline chloride as high as 240 m*M* did not have an observable effect. Conversely, it may be interpreted that the requirement for sodium in HE formation in these experiments was satisfied.

A second possibility might be inhibition of exocytotic processes responsible for the formation of the HE. However, there is currently no evidence to suggest that the exocytosis of the cortical vesicles associated with the elevation and transformation of the HE was inhibited in choline substituted seawater. When Tris was used as a sodium substitute, ring vesicle exocytosis was inhibited, as evidenced by the number of vesicles remaining in the cortical cytoplasm. On the other hand, the dense vesicles, which are the first to undergo exocytosis, were not observed in the egg cortex. Because the first phase of HE assembly was not observed in Tris-treated eggs, the fate of the dense vesicles is unclear.

Our data here demonstrate a significant effect of the ionic composition of seawater on the formation of the HE in *S. ingentis* and *T. similis*. These observations on HE assembly and elevation in the penaeoidean shrimp are consistent with data from the sea urchin system suggesting a role for sodium in the regulated assembly of an extracellular matrix.

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